ELSEVIER

Comparative Biochemistry and Physiology Part B 128 (2001) 647–659

CBP

www.elsevier.com/locate/cbpb

Discovery of novel trimethylalkanes in the internal hydrocarbons of developing pupae of *Heliothis virescens* and *Helicoverpa zea*

Dennis R. Nelson*

Biosciences Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, State University Station, Fargo, ND 58105, USA

Received 2 May 2000; received in revised form 29 October 2000; accepted 1 November 2000

Abstract

Novel trimethyl-branched alkanes which eluted with the monomethylalkanes were identified in the internal lipids of *Helicoverpa zea* but were not present in *Heliothis virescens*. Their structures were unique in that the first methyl branch occurred on carbon 2 and the 2nd and 3rd methyl branch points were separated by a single methylene. Novel trimethylalkanes identified from their chemical ionization and electron impact mass spectra were 2,18,20-trimethyltetratriacontane, 2,18,20-trimethylhexatriacontane, and 2,24,26-trimethyldotetracontane. Previous reports did not find these trimethylalkanes in the cuticular surface lipids of larvae, pupae or adults of either species. The internal pupal hydrocarbons of *H. virescens* and *H. zea* amounted to 123 μg and 304 μg per pupa, respectively. They consisted of *n*-alkanes (8 and 4%, respectively) and methyl-branched alkanes (88 and 94%, respectively). The *n*-alkanes ranged in chain length from approximately 21 to 35 carbons and the methyl-branched alkanes from approximately 26 to 55 carbons vs. methyl-branched alkanes from 28 to 37 carbons previously reported for hydrocarbons from the pupal cuticular surface. The major *n*-alkane was heptacosane (3.3 and 1.2%, respectively, in *H. virescens* and *H. zea*). The major methyl-branched alkanes in *H. virescens* were methylhentriacontane (15%), methyltritriacontane (12%) and dimethyltritriacontane (10%), and in *H. zea* were methylnonacosane (17%), dimethylnonacosane (9%) and methylhentriacontane (20%). Except for the novel trimethylalkanes, the methylalkane branch points were predominantly on odd-numbered carbons as has been reported for these and other species.

Keywords: Methyl-branched hydrocarbons; Cuticular hydrocarbons; Internal hydrocarbons; Lepidoptera; Pupae; *Heliothis virescens*; *Helicoverpa zea*

1. Introduction

Hydrocarbons are a common component of the surface lipids of all life stages of arthropods and in some cases are the major component (Blomquist et al., 1987; Lockey, 1988; Nelson, 1993; Nelson and Blomquist, 1995). They have found frequent use as chemotaxonomic fingerprints/characters to distinguish closely related species, usually in the adult stage, but may also be applicable to distinguish larvae before visible species-specific characteristics have developed (Carlson and Yocum, 1986; Nelson and Buckner, 1995). In previous studies, the differences in the cuticular surface hydrocarbons of larvae (Nelson

* Corresponding author. Tel.: +1-701-239-1286; fax: +1-701-239-1271.

E-mail address: dennis_nelson@ndsu.nodak.edu (D.R. Nelson).

1096-4959/01/$ - see front matter
PII: S1096-4959(00)00336-5

and Buckner, 1995), pupae (Buckner et al., 1996) and adults (Carlson and Milstrey, 1991) of *H. virescens* and *H. zea* have been characterized. Differentiation of these two species is of value as they attack several of the same crops and in the young larval stages can not be distinguished.

Hydrocarbons are also found in the hemolymph and among the internal tissues (Baker et al., 1963; Acree et al., 1965; Coudron and Nelson, 1981; Katase and Chino, 1984; Dwyer et al., 1986; Ahmad et al., 1989; Nelson et al., 1989). They are the source of the cuticular surface hydrocarbons; it appears that the hydrocarbons present on the cuticular surface of a particular stage were synthesized internally by the previous developmental stage (de Renobales et al., 1988). However, there is a selective transfer of internal hydrocarbon components from the internal sources to the cuticular surface. In the female housefly, tricosene, the female sex pheromone, does not appear on the cuticular surface until the female is sexually mature, even though tricosene may be a major component of the internal hydrocarbons (Adams and Nelson, 1990), and its appearance on the surface is under hormonal control (Adams et al., 1995).

In this paper we report on the composition and identification of the internal hydrocarbons of the pupal stage and show that *H. zea* has a unique series of novel trimethylalkanes with a bimodal distribution that chromatograph with the monomethylalkanes. These novel trimethylalkanes have not been found in the cuticular surface lipids of larvae (Nelson and Buckner, 1995), pupae (Buckner et al., 1996) or adults (Carlson and Milstrey, 1991) indicating a specific selectivity in the transport of internal hydrocarbons to the cuticular surface.

2. Materials and methods

2.1. Insects

Insects used were reared as previously described (Nelson and Buckner, 1995). All pupae were analyzed at third to half completion of the pupal stage, which we have termed mid-stage pupae, when biosynthesis of methyl-branched lipids is occurring (de Renobales et al., 1988, 1989; Nelson et al., 1990; Guo et al., 1992).

2.2. Lipid extraction

As previously described (Nelson et al., 1999a), surface lipids were removed by extracting each group of 12 pupae by stirring in 10 ml $CHCl_3$ for 30 s. The pupae were then homogenized in a Potter–Elvehjem tissue grinder with a teflon pestle in 25 ml $CHCl_3$:MeOH (C:M) (2:1). Solvent was removed on a flash evaporator, and the sample chromatographed on a silica gel thin-layer plate, developed in hexane/diethylether/formic acid (80:20:1), and the lipid bands located with iodine vapors and/or by placing the plate over a light box. Silica gel containing the hydrocarbon fraction was scraped into champagne tubes or sulfur absorption tubes and the hydrocarbons were eluted with 4 bed-volumes of hexane.

2.3. Chromatography and structural identification

CGC-MS (capillary gas chromatography-mass spectrometry) was performed on a Hewlett–Packard (H–P) 5890A gas chromatograph equipped with a pressure programable cool on-column injection port. The column consisted of a 1-m retention gap connected to a 12.5 m × 0.2 mm capillary column of crosslinked dimethyl silicone Ultra 1 (H–P), and was coupled to a H–P 5970B quadrupole mass selective detector. The carrier gas was He and the initial column temperature was between 150 and 200°C. The oven was programmed to 320°C at 3 or 4°/min and held at 320°C for 20–120 min. The system was controlled and data collected with an H–P 59970C computer. Total ion current values were transferred to Excel® for the preparation of CGC-MS elution traces. The graphics files were then imported into Freelance® for final editing and labeling. Quantitation and calculation of percent composition was done using *n*-alkane standards as described (Nelson et al., 1999b). Mass spectra were interpreted as previously described (Nelson, 1978; Blomquist et al., 1987; Nelson, 1993; Bernier et al., 1998; Carlson et al., 1998).

CI-CGC-MS (chemical ionization CGC-MS) was performed on a VG AutoSpec X042 operated at 45 eV. Gas chromatography conditions were as described above except that methane was the carrier gas. CI mass spectra were interpreted as previously described (Howard et al., 1980).

Table 1
Percent composition of the hydrocarbons of the internal lipids of developing pupae of *Heliothis virescens* and *Helicoverpa zea*

CGC-MS Peak[a]	Percent Composition[b]		Components[c]
	H. virescens	*H. zea*	
21	T	t	Henicosane
22	T	t	Docosane
23	T	t	Tricosane
24	0.8 ± 0.4	t	Tetracosane
25	T	t	Pentacosane
26	T	t	Hexacosane
26A	T	t	Methylhexacosane
26A′	0.8 ± 0.1	t	2-Methylhexacosane
27:1	t	–	Heptacosene
27	3.3 ± 0.4	1.2 ± 0.1	Heptacosane
27A	0.8 ± 0.1	0.5	Methylheptacosane
27A′	t	t	4- and/or 2-Methylheptacosane
27A′	0.8 ± 0.1	0.5 ± 0.1	3-Methylheptacosane
28	0.7	t	Octacosane
28A	0.3	0.7 ± 0.1	15-, 14-, 13*-, 12-, 11- and 10-Methyloctacosanes
28A′	3.2 ± 0.1	t	2-Methyloctacosane
28B	?	1.2	4- and 2-mono-, 9,13- and 8,12-di- and 3-monomethyloctacosanes
29:1	t	–	Nonacosene
29	1.2 ± 0.1	0.6	Nonacosane
29A	4.3 ± 0.1	16.9 ± 0.6	13*, 11-, 9-, 7- and 5-Methylnonacosanes
29B	t	8.7 ± 0.3	9,13**- and 7,13-Dimethylnonacosanes
29A′	0.7 ± 0.1	6.3 ± 0.4	3-Methylnonacosane
30:1	t	–	Triacontene
29B′	t?	0.7	3,7-Dimethylnonacosane
30A	1.7 ± 0.1	2.7	14-, 13-, 12-, 11-, 10- and 6-Methyltriacontanes
30A′	0.6	1.1 ± 0.1	2-Methyltriacontane
31	t	0.3	?
31A	15.0 ± 0.6	20.1 ± 0.3	15-, 13**-, 11*-, 9-, 7- and 5-Methylhentriacontanes
31B	4.3 ± 0.1	–	13,17- and 11,19-Di- and 3-mono-methylhentriacontanes
31B	–	5.8 ± 0.3	9,13- and 7,11-Di- and 3-mono-methylhentriacontanes
31B′	0.7 ± 0.1	0.8	5,19-, 5,17-, 5,15- and 5,13-Dimethylhentriacontanes
32	t	0.6	?
32A	2.7 ± 0.1	1.4 ± 0.1	16 to 7-Methyldotriacontanes
32B	1.5	–	14,18-, 13,17-Dimethyldotriacontanes and isomers with 5, 7 and 9 methylenes between branch points
32B	–	0.7	14,18*-, 13,17-, 10,14- and 9,13-Dimethyldotriacontanes
33	t	0.2	?
33A	12.4 ± 0.4	–	15-, 13- and 11*-Methyltritriacontanes
33A	–	3.6 ± 0.4	17*-, 15-, 13-, 11-, and 9-Methyltritriacontanes
33B	9.9 ± 0.3	–	15,19-, 13,17-, 13,19-, 13,21*- and 11,21-Dimethyltritriacontanes
33B	–	2.4 ± 0.2	15,19*-, 13,17*- and 11,15-Dimethyltritriacontanes
34:1	t	–	Tetratriacontene
34A	0.9	–	14-, 13-, 12-, 11*- and 10-Methyltetratriacontanes
34A	–	0.3	17-, 16-, 15-, 14- and 13-Methyltetratriacontanes
34B	0.8	–	13,21- and 11,??-Dimethyltetratriacontanes
34B	–	0.4	15,19- and 14,18-Dimethyltetratriacontanes
35A	1.9 ± 0.2	–	15-, 13- and 11*, **-Methylpentatriacontanes
35A,34C′	–	1.0 ± 0.2	17- and 15-Methylpentatriacontanes and 2,18,20-trimethyltetratriacontane
35B	2.1 ± 0.2	–	15,19-, 13,23- and 11,??-Dimethylpentatriacontanes
35B	–	2.0 ± 0.4	15,19*-, 13,??-, 11,??- and 9,??-Dimethylpentatriacontanes
35C/36:1	t	t	?
36A,35C′	–	t	18-, 17- and 16-Methylhexatriacontanes and Trimethylpentatriacontane
36B	–	t	15,19- and 16,20-Dimethylhextriacontanes

Table 1 (*Continued*)

CGC-MS Peak[a]	Percent Composition[b]		Components[c]
	H. virescens	*H. zea*	
37A,36C′	0.2	0.3	19-, 17-, 15- and 13-Methylheptatriacontanes and 2,18,20-trimethylhextriacontane
37B	1.4 ± 0.1	–	11,??-, 9,19*- and 11,??-Dimethylheptatriacontanes
37B	–	1.2 ± 0.1	17,21-, 15,19-, 13,??-, 11,??-, 9,19- and 7,??-Dimethylheptatriacontanes
37C	?	0.4	9,13,17-, 9,13,19- and 7,11,??-Trimethylheptatriacontanes and ??
37C	–	0.2	5,9,??-, 5,11,??- and 5,13, ??-Trimethylheptatriacontanes
38A	0.2	0.2	19-, 18- and 17-Methyloctatriacontanes and ?
38B	1.2	–	12,20-, 11,19- and 10,18-Dimethyloctatriacontanes
38B	–	0.9	12,20- and 10,20*-Dimethyloctatriacontanes
38C	–	t	8,12,18- and 8,12,20-Trimethyloctatriacontanes
39A	0.6	0.5	19-Methylnonatriacontane
39B	5.3 ± 0.3	4.8 ± 0.5	11,19- and 9,19-Dimethylnonatriacontanes + $11,21^{H.v.}$ and $13,21^{H.z.}$
39C	t	1.0	9,13,??-, 7,11,19- and 5,9,??-Trimethylnonatriacontanes
40A	t	0.2	20-Methyltetracontane and ?
40B	1.0	0.7 ± 0.1	12,20- and 10,20-Dimethyltetracontanes
41A	0.1	t	21- and 19-Methylhentetracontanes and ?
41B	1.7 ± 0.1	0.9	11,21- and 11,19-Dimethylhentetracontanes
42B	t	t	?
43A,42C′	t	t	18- and 17-Methyltritetracontane and 2,24,26-trimethyldotetracontane
43B	0.5	0.5	17,21-, 15,??-, 13,??- and 11,23-Dimethyltritetracontanes
44A	?	t	17-Methyltetratetracontane
44B	t?	t	17,??- and 16,??-Dimethyltetratetracontanes
45A,44C′	0.2	0.3	19- and 17-Methylpentatetracontanes and two trimethyl′s
45B	2.3 ± 0.1	–	17,27-, 15,25- and 13,23-Dimethylpentatetracontanes
45B	–	1.6 ± 0.3	17,21*-, 15,??-, 13,??- and 11,??-Dimethylpentatetracontanes
46A	t?	t	18- and 17-Methylhexatetracontanes
46B	t?	t	18,22-, 17,21-, 16,20*- and 15,19-Dimethylhexatetracontanes
47A,46C′	t?	t	?
47B	2.3 ± 0.1	–	15,23-, 15,??-, 13,17-, 13,23- and 13,??-Dimethylheptatetracontanes
47B	–	1.0 ± 0.1	17,21-, 15,??-, 13,??- and 11,??-Dimethylheptatetracontanes
48B	0.1	t	?
49A	T	t	?
49B	0.9 ± 0.1	0.4	13,??-Dimethylnonatetracontane

[a] The CGC-MS peak numbers correspond to those marked in Fig. 1. The number represents the number of carbon atoms in the backbone (carbon chain) of the methyl-branched alkane. The letters A, B, and C indicate one, two and three methyl branches, respectively. A letter with a prime symbol means that one of the methyl branches is near the end of the carbon chain, e.g., on carbon 2, 3 or 4 which causes the trimethylalkane to elute with the internally branched monomethylalkane with a carbon chain backbone one carbon longer.

[b] The values for percent composition are the mean ± standard deviation. Values less than 0.2% are listed as 't'. Standard deviations less than 0.1 are not listed with the mean values. *H. virescens* and *H. zea* pupae had 123 ± 8 and 304 ± 28 μg/pupa, respectively, of internal hydrocarbons.

[c] The compounds were identified from their electron impact mass spectra. The numbers indicated the positions of the methyl branches on the backbone of the molecule. A '?' means that the compound could not be identified from its mass spectrum and that its identity is estimated based on its elution position. For *n*-alkane positions, 2,x-, 3,x- and 4,x-dimethylalkanes would elute at approximately the same position; none were detected. Methyl branch positions that could not be established with certainty are indicated by '??'. If the major isomer could be estimated, it is indicated with an '*'. Where the major isomer differed between *H. virescens* and *H. zea*, the major isomer in *H. virescens* is indicated by one '*' and in *H. zea* by two '**'.

3. Results

3.1. CGC-MS chromatography

CGC-MS analysis of the hydrocarbon fractions from the internal lipids of mid-stage pupae of *H. virescens* and *H. zea* showed that the two species had similar profiles and that the major components were long-chain methyl-branched alkanes (Fig. 1 and Table 1). In both species, the major *n*-alkane was heptacosane (C27). However, the methylalkanes differed between the species: *H. zea* had major peaks at 29A and 29B,A′, and at 31A and 31B,A′ (Fig. 1A), while *H. virescens* had

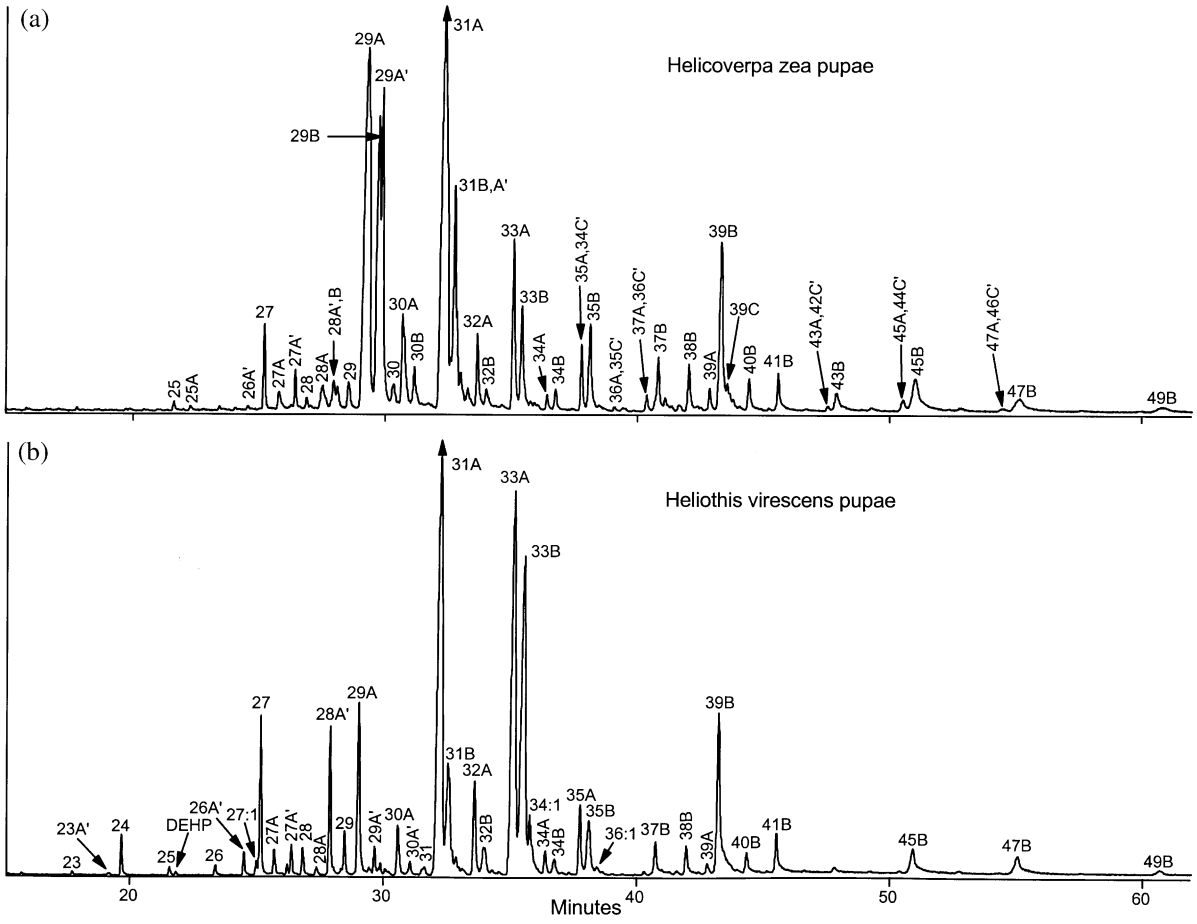

Fig. 1. CGC-MS total ion current chromatograms of the hydrocarbon fractions of the pupal total lipid extract: (a) *Helicoverpa zea*, and (b) *Heliothis virescens*. The CGC-MS peak numbers correspond to the number of carbon atoms in the backbone (carbon chain) of the hydrocarbon. The letters A, B and C, indicate one, two or three methyl branches, respectively. A prime mark indicates one of the methyl branches was near the end of the chain, i.e. on carbon 2, 3 or 4.

peaks 2 carbons longer at 31A and 33A, and at 33B (Fig. 1B). The two major monomethyl-branched alkanes in *H. zea* were methylnonacosane, 29A (17%) and methylhentriacontane, 31A (20%). The major dimethyl-branched alkanes were dimethylnonacosane, 29B (9%), in *H. zea*, and dimethyltritriacontane, 33B (10%) in *H. virescens*. Although the hydrocarbon components are similar for both species, the species are readily distinguishable from the CGC-MS profiles.

Most of the mass spectra were readily interpreted unless the mixture of isomers produced too much spectral overlap. However, the mass spectra of the A series from *H. zea*, beginning with 35A had a diagnostically significant odd-mass ion(s) in addition to the even-mass ions characteristic of internally branched monomethylalkanes. Diagnostic odd-mass ions arise from secondary carbonium ions containing a second methyl branch on a carbon other than carbon 2 (Blomquist et al., 1987; Nelson, 1978). Interestingly, the appearance of the diagnostic odd-mass ions appeared to be bimodal through the A series from *H. zea*. They were present in CGC-MS peaks 35A, 36A and 37A; were ambiguous in 38A, 40A and 41A; were not present in 39A; and again occurred in peaks 43A, 44A, 45A, 46A, 47A and 49A [peaks 42A and 48A were too minor to determine (Fig. 1a)]. Thus, these CGC-MS peaks appeared to have a multiple branched component(s) in addition to the internally branched monomethylalkane(s).

3.2. Mass spectra of the A and putative C′ series

Peak 35A of *H. zea* appeared to be a mixture of 17- and 15-methylpentatriacontanes based on the ions at *m/z* 252:280 and 224:308, respectively (Fig. 2a). A diagnostic ion at *m/z* 491 corresponding to M-15 for a monomethylpentatriacontane was also present. The greater intensity

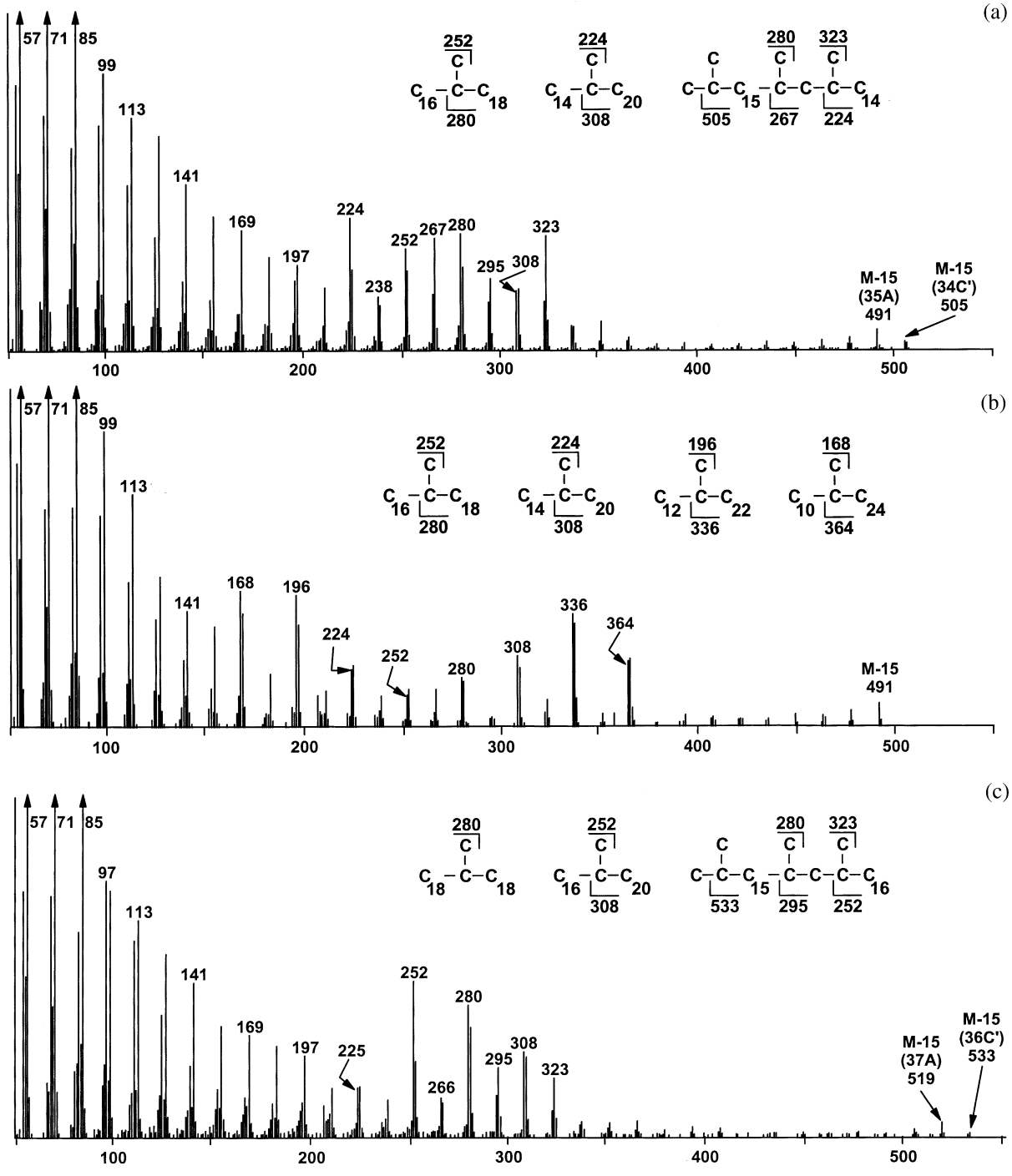

Fig. 2. Mass spectra of: (a) CGC-MS peak 35A,34C′ from *H. zea*; (b) CGC-MS peak 35A from *H. virescens*; (c) CGC-MS peak 37A,36C′ from *H. zea*; (d) CGC-MS peak 39A from *H. zea*; (e) CGC-MS peak 43A,42C′ from *H. zea*.

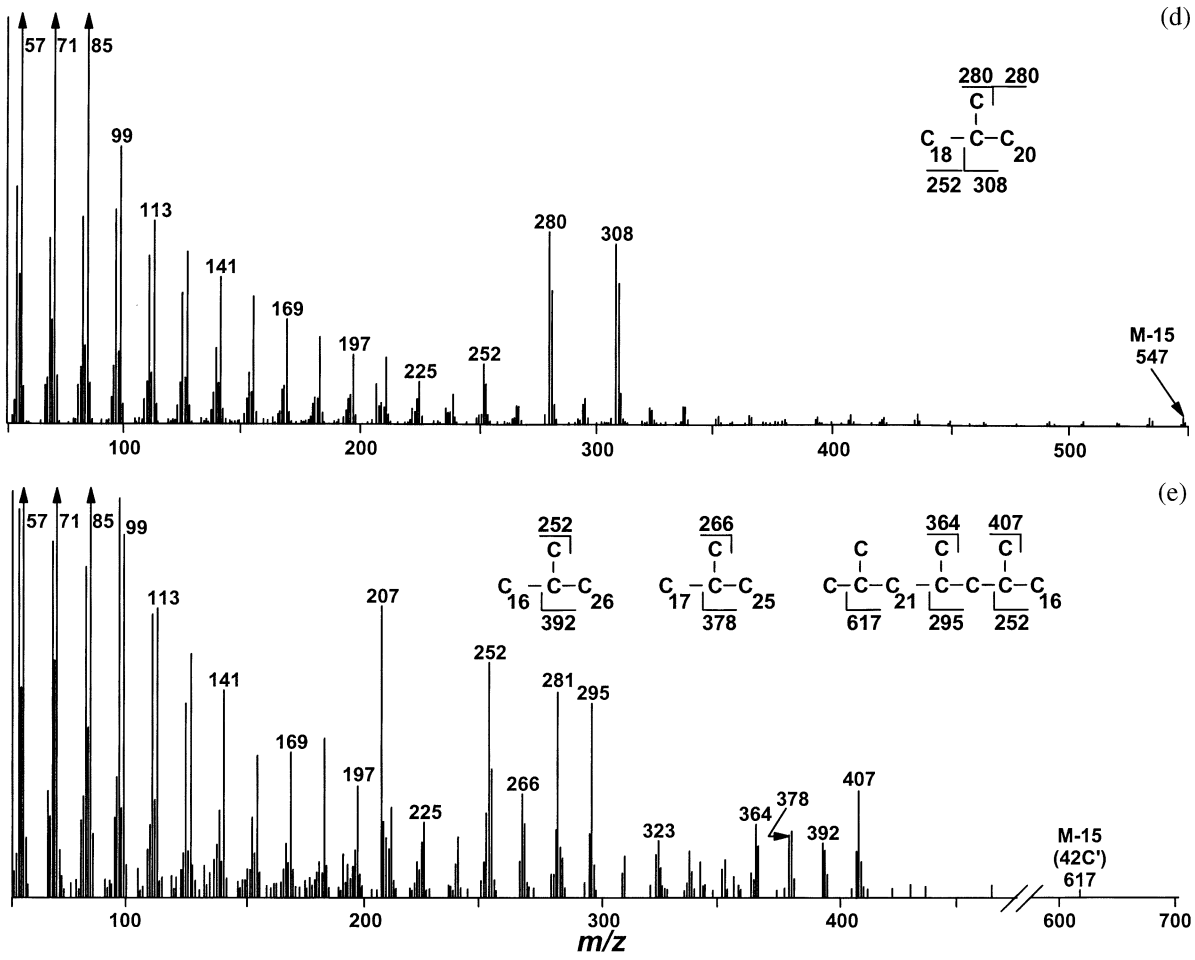

Fig. 2. (*Continued*).

of the ion at m/z 280 than that at 252 indicates there is another source for the ion at m/z 280 in addition to the 17-methyl isomer. Also, the much greater intensity of m/z 224 compared to m/z 308, indicates that m/z 224 is also derived from some other methylalkane in addition to the 15-methyl isomer.

In comparison, peak 35A from *H. virescens* is readily interpreted as being solely that of a mixture of internally branched monomethylpentatriacontanes; 17-, 15-, 13- and 11-methylpentatriacontanes (Fig. 2b). The presence of the 17-methyl isomer was more evident in earlier scans. Also, the 17- and 15-methylisomers were only minor components of 35A in *H. virescens*, but were the only two internally branched monomethyl components in *H. zea*. Odd-mass ions of diagnostic intensity were not present in the mass spectra of 35A from *H. virescens* (Fig. 2b).

The mass spectra of peak 35A from *H. zea* was notable in that it had odd-mass ions of marked intensity at m/z 267 and 323 indicating the presence of a component with more than one methyl branch (Fig. 2a). The fact that the unknown component was eluting with 35A indicated that it might correspond to 34C′, a trimethyl-branched alkane of 37 carbons in which one of the methyl branches is near the end of the carbon chain, as the number of methyl branches and their positions are known to have an effect on the elution time. This conclusion was supported by the predicted M-15 ion for such a trimethylalkane at m/z 505 (Fig. 2a) and CI-MS M-1 ions at m/z 505 and 519 for 35A and the putative 34C′, respectively (data not shown). Therefore, the peak 35A from *H. zea* was designated as 35A,34C′.

None of the structures with 3 or 5 methylenes between the branch points frequently found in

insects satisfactorily accounted for, in particular, the ion at m/z 267 in the mass spectrum of peak 35A,34C′ from *H. zea* (Fig. 2a). The most likely isomer for the putative 34C′ from *H. zea* was concluded to be 2,18,20-trimethyltetratriacontane which is unique because it has the two internal methyl branch points, on carbons 18 and 20, separated by only one methylene group. This isomer satisfactorily accounts for the major diagnostic odd-mass ions at m/z 267 and 323, and for the greater intensity of m/z 224 compared to that of the ion at m/z 308 derived from 15-methylpentatriacontane. The diagnostic ion for the 2-methyl-branched secondary carbonium ion occurs at m/z 280 rather than m/z 281. It has been shown that when the second methyl branch is on carbon 2 that it does not suppress the loss of a hydrogen atom from the branched secondary carbonium ion (Pomonis et al., 1989). The ion at m/z 351 indicates the presence of a trace amount of an isomer in which the 3rd methyl group is on carbon 22.

CGC-MS peaks 36A,35C′ and 37A,36C′ from *H. zea* also had both even-mass and odd-mass ions of such an intensity to be diagnostic and indicate the presence of both A and C′ components. In peak 37A,36C′, the ion at m/z 280 is formed by the fragmentation of 19-methylheptatriacontane on either side of the methyl branch point (Fig. 2c). The fragmentation of 17-methylpentatriacontane forms ions at m/z 252 and 308. The much greater intensity of m/z 252 compared to that of m/z 308 indicates that there is another source for m/z 252. One source is the 18-carbon straight-chain tail from the 19-methyl-isomer. Another source is the trimethylalkane, 2,18,20-trimethylhexatriacontane, which also accounts for the odd-mass ions at m/z 295 and 323 as well as for the intensity of m/z 252. A putative M-15 ion also is present at m/z 533 for the trimethylalkane. CI-MS verified the presence of the trimethylalkane by a M-1 ion at m/z 547 in addition to the M-1 ion at m/z 533 for 37A (data not shown). Because of the near symmetry of the two internal methyl branches on the carbon chain backbone of 36C′, the alternate structure of a 2,16,18-isomer also would form the identical secondary carbonium ions. Thus, it is not possible to determine whether one or the other, or both are present. However, the structure of a 2,18,20-isomer is preferred because in a homologous series the distance along the carbon backbone tends to increase, not decrease.

The first C′ series (of an apparent bimodal series) of trimethylalkanes appears to have ended by the time peak 39A had eluted. CGC-MS peak 39A is clearly a single internally branched monomethylalkane (Fig. 2d). There was no indication of the presence of a 38C′ component. CGC-MS peak 39A from both *H. virescens* and *H. zea* consisted of the same single component, 19-methylnonatriacontane.

As the chain length increased further, diagnostic odd-mass ions were again apparent in CGC-MS peak 43A,42C′ (Fig. 2e). Internally branched monomethylalkanes were indicated by the ions at m/z 266 and 378 for the 18-methyl isomer, and at m/z 252 and 392 for the 17-methyl isomer. Again, the much greater intensity of m/z 252 than 392 indicates that there is another source for 252, and the diagnostic odd-mass ions indicate a component with more than one methyl group. The trimethylalkane, 2,24,26-trimethyltritetracontane readily accounts for the large m/z 252 and for the other diagnostic even-mass (364) and odd-mass (295 and 407) ions. The ions at m/z 207 and 281 are largely from column bleed. CI-MS showed the M-1 ions expected for a mixture of 43A and 42C′ (data not shown). This appears to be the only structure that can be drawn to account for the observed diagnostic ions. This mass spectrum appears to establish the presence of trimethylalkanes with a methyl branch on carbon 2, with two internal methyl branches separated by one methylene group and lends support to the previous interpretations for CGC-MS peaks 34C′ (Fig. 2a) and 36C′ (Fig. 2c).

Only one CGC-MS peak, peak 36A,35C′ (mass spectrum not shown), had a C′ component with an odd number of carbons in the backbone of the molecule. The major monomethylakanes were obvious, 18-, 17- and 16-methylhexatriacontanes (Table 1). Although odd-mass ions were evident at m/z 323 and 337, the mass spectra were too weak to determine which of the other ions were derived from a trimethyl-branched alkane. The ion at m/z 491 was of increased intensity relative to the adjacent clusters of ions due to carbon-carbon bond cleavage. This ion could only be formed by a trimethylalkane in which the first methyl branch was on carbon 4, i.e. a 4,x,y-trimethylalkane.

3.3. Mass spectra of the B and C series

The B and C series of methyl-branched hydrocarbons had a similar CGC-MS profile for both species. However, the mass spectra showed that the structures of the dimethylalkane isomers differed more in *H. virescens* than in *H. zea*. The dimethylalkane branch points in peaks 31B and 33B from *H. virescens* were separated by 3, 5, 7 and 9 carbons (Table 1). These same peaks from *H. zea* had the methyl branch points separated by 3 carbons in all isomers. It was not until the chain length had increased to peak 37B that *H. zea* hydrocarbons again contained a dimethyl isomer with more than 3 carbons between the branch points.

An unusual feature was the appearance of 39B in notable amounts in both species. The major isomers were identical in both *H. virescens* and *H. zea*, i.e. 11,19- and 9,19-dimethylnonatriacontanes. However, *H. virescens* also had a trace amount of the 11,21-isomer while *H. zea* had a small amount of the 13,21-isomer. In 39B, both species had dimethylalkanes separated by 7 and 9 methylene groups (Table 1).

Thus, the two species could be differentiated both on their CGC-MS profiles as well as the structures of the isomers of their dimethylalkanes. A comparison of structures of the trimethylalkane series could not be made because only trace amounts, if any, were present in *H. virescens*.

4. Discussion

The composition of hydrocarbons to differentiate species was used as early as 1963 (Eglington and Hamilton, 1963). Hydrocarbon profiles of insects, and/or structures of methyl-branched alkane components, have been used to differentiate closely related species (Lockey, 1988, 1991; Nelson and Blomquist, 1995). Our experience as well as that of others (Ogden et al., 1998) has shown that a capillary gas chromatography system equipped with a cool on-column injector gives the best results for either hydrocarbons, wax esters,

Table 2
Comparison of the composition of the major components of hydrocarbons from the cuticular surface of larvae, pupae and adults, and from the internal lipids of pupae

CGC-MS Peak no.[a]	Percentage Composition							
	Heliothis virescens				*Helicoverpa zea*			
	Larvae[b]	Pupae[c]	Pupae (internal)	Adults[d]	Larvae[b]	Pupae[c]	Pupae (internal)	Adults[d]
25	t	22	t	1	1	2	t	3
27	15	8	3	3	13	13	1	9
28A′	5	4	3	1	–	–	–	t
29	4	3	1	1	3	33	1	4
29A	2	5	4	2	16	12	17	12
29B	t	–	t	1	8	7	9	10
31:1	–	–	t	?	5	–	–	?
31A	14	15	15	17	22	15	20	16
31B	1	3	4	7	5	3	6	5
33A	17	11	12	8	3	2	4	2
33B	9	5	10	16	1	1	2	3
37B	–	–	1	3	t	–	1	2
39B	–	–	5	13	–	–	5	6
41B	–	–	2	4	–	–	1	2
47B	4	–	2	2	–	–	–	2

[a] The CGC-MS peak numbers correspond to the number of carbon atoms in the backbone (carbon chain) of the hydrocarbon. The letters A and B, indicate one or two methyl branches, respectively. A prime symbol indicates the methyl branch is near the end of the carbon chain. The ':1' indicates one double bond.

[b] Data from Nelson and Buckner (1995).

[c] Data from Buckner et al. (1996).

[d] Data estimated from Carlson and Milstrey (1991). In their procedure, any alkenes that may have been present would have been removed before analysis.

or a mixture of the two. The hydrocarbon composition for both *H. virescens* and *H. zea* was similar for all stages, whether from the internal pupal contents or from the cuticular surface (Table 2). Unsaturated components were either absent or only present in minor amounts. *H. virescens* tended to have a greater percentage of longer chain-length components than did *H. zea*. In both species, hydrocarbons with carbon chain backbones of 27, 29, 31 and 33 tended to predominate. However, there were two *n*-alkanes that were the major components in the cuticular surface hydrocarbons of pupae: C25 on *H. virescens* pupae and C29 on *H. zea* pupae. Even in those two instances, however, methyl-branched hydrocarbons were more than 50% of the total.

Although elution profiles from gas chromatography as well as high performance liquid chromatography are useful in differentiating species, the differentiation can be taken further by examining the mass spectra of the eluting peaks. In the hydrocarbons from *H. zea*, two major diagnostic odd-mass ions were present in the mass spectra of all CGC-MS peaks of the A series which indicated a C′ component was also present. The presence of the C′ component was not apparent in the A series of the internal hydrocarbons of *H. zea*. Also, a number of the B series components showed differences in the number of carbons separating the methyl branch points between *H. virescens* and *H. zea*.

The position of the methyl branch on the carbon chain can have a large influence on the gas chromatographic equivalent chain length (Mold et al., 1966). The effect of single, and the additive effects of multiple methyl branches, on the equivalent chain length of methylalkanes have been determined in a number of studies on insects (Nelson, 1978; Blomquist et al., 1987; Kaib et al., 1993; Provost et al., 1993; Brown et al., 1996a,b; Howard and Infante, 1996; Brown et al., 1997; Bernier et al., 1998; Carlson et al., 1998). The first published mass spectra of terminally branched trimethylalkanes were for a homologous series of 3,7,11- and 4,8,12-trimethylalkanes from *Atta Colombia* (Martin and MacConnell, 1970). These studies all indicate that a trimethylalkane with one of the methyl branches near the end of the carbon chain backbone would elute with internally branched monomethylalkanes having a carbon chain backbone one carbon greater in length than that of the trimethylalkane. Thus, 2,18,20-trimethyltetratriacontane (37 carbon trimethylalkane with a 34 carbon chain backbone) eluted with 19-methylpentatriacontane (36 carbon monomethylalkane with a 35 carbon chain backbone).

Terminally branched trimethylalkanes (C′), i.e. one of the methyl groups is on carbon 2, 3 or 4 of the carbon chain, have been characterized by GC-MS but in most reports the mass spectra were not published. A series with the first methyl branch point at carbon 3 was reported in *Drepanotermes* sp. (Brown et al., 1996a,b). These trimethylalkanes, e.g. 3,7,11-trimethylhentriacontane (equivalent chain length; ECL = 32.38), had an ECL only slightly larger than for the internally branched monomethylalkane, monomethyldotriacontane (ECL = 32.33) causing them to overlap or coelute during chromatography, especially as the carbon chain increased in size. A series with the first methyl branch point on carbon 4 was reported in *Messor barbarus* (Provost et al., 1994). Two series, one with the first methyl branch point on carbon 3 and the other with the methyl branch point on carbon 4 were reported from *Glossina,* and the ECL measured predicts that they would overlap in their elution with an internally branched monomethylalkane (Sutton and Carlson, 1997), particularly as the carbon chain increased in size.

The structures deduced from the mass spectra for the three peaks with trimethyl-branched components in this study were consistent with a single methylene group separating the methyl branch points of the two internal methyl groups. The finding of a single methylene between branch points is relatively rare although there are a few reports. A short-chain trimethylalkene, 3,5,7-trimethyl-2,4,6,8-undecatetraene, with the methyl branch points separated by a single methine group has been characterized as a pheromone in several species of *Carpophilus* (Petroski et al., 1994). An internally branched trimethylalkane in which all the methyl branch points are separated by one methylene group, 13,15,17-trimethylnonacosane, was identified in *C. formosanus* (McDaniel, 1990), and the mass spectrum was later published (Haverty et al., 1996b).

Dimethylalkanes with one methylene between the branch points were earlier reported from *Popillia japonica* (Bennett et al., 1972), *Solenopsis invicta* and *S. richteri* (Lok et al., 1975), *Pogonomyrmex rugosus* (Regnier et al., 1973), and

Triatoma infestans (Juárez and Brenner, 1985). However, no mass spectral data were presented, and/or structures were indicated as 'tentative', or could not be confirmed (Blomquist et al., 1987; Nelson, 1978).

Dimethylalkanes with one methylene between the methyl branch points have been identified and the mass spectra published for 13,15-dimethylheptacosane and 13,15-dimethylnonacosane in *S. invicta*, and for 13,15-dimethylheptacosane in *S. richteri* (Nelson et al., 1980), and confirmed by the synthesis of both homologues (Thompson et al., 1981). A homolgous series of 11,13-dimethyalkanes and the compound 15,17-dimethylhentetracontane were reported from *Reticulitermes* sp. (Haverty et al., 1996a). A report on hydrocarbons from *Reticulitermes* in Japan (Takematsu and Yamaoka, 1999) appears to list dimethylalkanes in the table of data, but this appears to be an editing error in the shorthand designation of the compounds as no reference is made to dimethylalkanes in the text and the listed ECL correspond more closely to monomethylalkanes. A series of 12,14-, 13,15- and 15,17-dimethylalkanes were identified in *Coptotermes formosanus* (Haverty et al., 1996b). Other reports of monomethylene interrupted methyl branch points are 11,13-dimethylhentriacontane, 13,15-dimethyltritriacontane and 13,15-dimethylpentatriacontane in *Myrmecophilus* sp. (Akino et al., 1996), and a series of 11,13- and 13,15-dimethylalkanes with carbon chain backbones from 25 to 33 carbons in some *Cataglyphis* species (Dahbi et al., 1996)

The present findings of the differences in the B isomers, and in particular the C′ components, demonstrates the value of using a mass spectrometer as the detector in gas chromatography. The results here, as well as in a number of the cited papers, show the large number of structures possible in a hydrocarbon sample, not only from a single species but from different tissues of the species. This, in addition to the differences in methylalkane structure between species, is useful for taxonomic purposes and biosynthetic studies.

Acknowledgements

Thanks to Charlotte L. Fatland for processing the samples and obtaining the electron impact mass spectra, to Margaret K. Lorentzsen for obtaining the CI mass spectra, and to Marcia M. Hagen for the *H. virescens* pupae. The use of trade, firm, or corporation names is for the information and convenience of the reader. Such use does not constitute an official endorsement by the U.S. Department of Agriculture or the Agricultural Research Service of any product to the exclusion of others that may be suitable.

References

Acree, F., Turner, R.B., Smittle, B.J., Burden, G.S., 1965. Hydrocarbons in haemolymph of cockroaches of different ages. J. Insect Physiol. 11, 905–910.

Adams, T.S., Nelson, D.R., 1990. The influence of diet on ovarian maturation, mating, and pheromone production in the housefly, *Musca domestica*. Invert. Reprod. Dev. 17, 193–201.

Adams, T.S., Nelson, D.R., Fatland, C.L., 1995. Effect of methylalkanes on male house fly, *Musca domestica*, sexual behavior. J. Insect Physiol. 41, 443–449.

Ahmad, S., Mackay, M.E., Blomquist, G.J., 1989. Accumulation of the female sex pheromone and its transfer to, and metabolism in, the male housefly, *Musca domestica L.*, during courtship and mating. J. Insect Physiol. 35, 775–780.

Akino, T., Mochizuki, R., Morimoto, M., Yamaoka, R., 1996. Chemical camouflage of myrmecophilous cricket *Myrmecophilus* sp. to be integrated with several ant species. Jpn. J. Appl. Entomol. Zool. 40, 39–46.

Baker, G.L., Vroman, H.E., Padmore, J., 1963. Hydrocarbons of the American cockroach. Biochem. Biophys. Res. Commun. 13, 360–365.

Bennett, G.A., Kleiman, R., Shotwell, O.L., 1972. Hydrocarbons in haemolymph from healthy and diseased Japanese beetle larvae. J. Insect Physiol. 18, 1343–1350.

Bernier, U.R., Carlson, D.A., Geden, C.J., 1998. Gas chromatography/mass spectrometry analysis of the cuticular hydrocarbons from parasitic wasps of the genus *Muscidifurax*. J. Am. Soc. Mass Spectrom. 9, 320–332.

Blomquist, G.J., Nelson, D.R., de Renobales, M., 1987. Chemistry, biochemistry, and physiology of insect cuticular lipids. Arch. Insect Biochem. Physiol. 6, 227–265.

Brown, W.V., Rose, H.A., Lacey, M.J., 1997. The cuticular hydrocarbons of the soil burrowing cockroach *Geoscapheus dilatatus* (Saussure) (Blattodea: Blaberidae: Geoscapheinae) indicate species dimorphism. Comp. Biochem. Physiol. 118B, 549–562.

Brown, W.V., Watson, J.A.L., Lacey, M.J., 1996a. A chemotaxonomic survey using cuticular hydrocarbons of some species of the Australian harvester

termite genus *Drepanotermes* (Isoptera: Termitidae). Sociobiology 27, 199–221.

Brown, W.V., Watson, J.A.L., Lacey, M.J., Morton, R., Miller, L.R., 1996b. Composition of cuticular hydrocarbons in the Australian harvester termite *Drepanotermes perniger* (Isoptera: Termitidae): Variation among individuals, castes, colonies and locations. Sociobiology 27, 181–197.

Buckner, J.S., Mardaus, M.C., Nelson, D.R., 1996. Cuticular lipid composition of *Heliothis virescens* and *Helicoverpa zea* pupae. Comp. Biochem. Physiol. 114B, 207–216.

Carlson, D.A., Bernier, U.R., Sutton, B.D., 1998. Elution patterns from capillary GC for methyl–branched alkanes. J. Chem. Ecol. 24, 1845–1865.

Carlson, D.A., Milstrey, S.K., 1991. Alkanes of four related moth species, Helicoverpa and Heliothis. Arch. Insect Biochem. Physiol. 16, 165–175.

Carlson, D.A., Yocum, S.R., 1986. Cuticular hydrocarbons from six species of tephritid fruit flies. Arch. Insect Biochem. Physiol. 3, 397–412.

Coudron, T.A., Nelson, D.R., 1981. Characterization and distribution of the hydrocarbons found in diapausing pupae tissues of the tobacco hornworm, *Manduca sexta* (L.). J. Lipid Res. 22, 103–112.

Dahbi, A., Cerdá, X., Hefetz, A., Lenoir, A., 1996. Social closure, aggressive behavior, and cuticular hydrocarbon profiles in the polydomous ant *Cataglyphis iberica* (Hymenoptera: Formicidae). J. Chem. Ecol. 22, 2173–2186.

Dwyer, L.A., Zamboni, A.C., Blomquist, G.J., 1986. Hydrocarbon accumulation and lipid biosynthesis during larval development in the cabbage looper, *Trichoplusia ni*. Insect Biochem. 16, 463–469.

Eglington, G., Hamilton, R.J., 1963. In: Swain, T. (Ed.), Chemical Plant Taxonomy. Academic Press, London, p. 187.

Guo, L., Nelson, D.R., Fatland, C.L., Blomquist, G.J., 1992. Very long-chain methyl-branched alcohols and their acetate esters in pupae of the southern armyworm, *Spodoptera eridania*: Identification and biosynthesis. Insect Biochem. Mol. Biol. 22, 277–283.

Haverty, M.I., Forschler, B.T., Nelson, L.J., 1996a. An assessment of the taxonomy of *Reticulitermes* (Isoptera: Rhinotermitidae) from the southeastern United States based on cuticular hydrocarbons. Sociobiology 28, 287–318.

Haverty, M.I., Grace, J.K., Nelson, L.J., Yamamoto, R.T., 1996b. Intercaste, intercolony, and temporal variation in cuticular hydrocarbons of *Coptotermes formosanus* Shirake (Isoptera: Rhinotermitidae). J. Chem. Ecol. 22, 1813–1834.

Howard, R.W., Infante, F., 1996. Cuticular hydrocarbons of the host-specific ectoparasitoid *Cephalonomia stephanoderis* (Hymenoptera: Bethylidae) and its host the coffee berry borer (Coleoptera: Scolytidae). Ann. Entomol. Soc. Am. 89, 700–709.

Howard, R.W., McDaniel, C.A., Nelson, D.R., Blomquist, G.J., 1980. Chemical ionization mass spectrometry; application to insect-derived cuticular alkanes. J. Chem. Ecol. 6, 609–623.

Kaib, M., Heinze, J., Ortius, D., 1993. Cuticular hydrocarbon profiles in the slave-making ant *Harpagoxenus sublaevis* and its hosts. Naturwissenschaften 80, 281–285.

Juárez, M.P., Brenner, R.R., 1985. The epicuticular lipids of *Triatoma infestans*-II. Hydrocarbon dynamics. Comp. Biochem. Physiol. 82B, 793–803.

Katase, H., Chino, H., 1984. Transport of hydrocarbons by haemolymph lipophorin in *Locusta migratoria*. Insect Biochem. 14, 1–6.

Lockey, K.H., 1988. Review – Lipids of the insect cuticle: origin, composition and function. Comp. Biochem. Physiol. 89B, 595–645.

Lockey, K.H., 1991. Insect hydrocarbon classes: implications for chemotaxonomy. Insect Biochem. 21, 91–97.

Lok, J.B., Cupp, E.W., Blomquist, G.J., 1975. Cuticular lipids of the imported fire ants, *Solenopsis invicta* and *richteri*. Insect Biochem. 5, 821–829.

Martin, M.M., MacConnell, J.G., 1970. The alkanes of the ant, *Atta Colombia*. Tetrahedron 26, 307–319.

McDaniel, C.A., 1990. Cuticular hydrocarbons of the Formosan termite *Coptotermes formosanus*. Sociobiology 16, 265–273.

Mold, J.D., Means, R.E., Stevens, R.K., Ruth, J.M., 1966. The paraffin hydrocarbons of wool wax. Homologous series of methyl alkanes. Biochemistry 5, 455–461.

Nelson, D.R., 1978. Long-chain methyl-branched hydrocarbons: occurrence, biosynthesis, and function. Adv. Insect Physiol. 13, 1–33.

Nelson, D.R., Buckner, J.S., 1995. The surface hydrocarbons of larval *Heliothis virescens* and *Helicoverpa zea*. Comp. Biochem. Physiol. 111B, 681–689.

Nelson, D.R., 1993. Methyl-branched lipids in insects. In: Stanley-Samuelson, D.W., Nelson, D.R. (Eds.), Insect Lipids: Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln, pp. 271–315.

Nelson, D.R., Blomquist, J.G., 1995. Insect waxes, Ch. 1. In: Hamilton, R.J., Christie, W.W. (Eds.), Waxes: Chemistry, Molecular Biology and Functions. The Oily Press Ltd., Dundee, Scotland, pp. 1–90.

Nelson, D.R., Fatland, C.L., Buckner, J.S., de Renobales, M., Blomquist, G.J., 1990. Biosynthesis of very long-chain methyl-branched alcohols, their acetate esters and hydrocarbons in pupae of the tobacco hornworm, *Manduca sexta*. Insect Biochem. 20, 821–827.

Nelson, D.R., Fatland, C.L., Howard, R.W., McDaniel, C.A., Blomquist, G.J., 1980. Re-analysis of the cuticular methylalkanes of *Solenopsis invicta* and *S. richteri*. Insect Biochem. 10, 409–418.

Nelson, D.R., de Renobales, M., Dwyer, L.A., Zamboni, A.C., Fatland, C.L., Blomquist, G.J., 1989. Novel very long-chain methyl-branched alcohols and their esters, and methyl-branched alkanes in pupae of the cabbage looper, *Trichoplusia ni* (Hubner). Insect Biochem. 19, 197–208.

Nelson, D.R., Fatland, C.L., Adams, T.S., 1999a. Very long-chain methyl-branched alcohols and their acetate esters in pupal internal lipids of developing Coleoptera, *Anthonomus grandis* and *Cylindrocopturus adspersus*, and Diptera, *Cochliomyia hominivorax* and *Musca domestica*. Comp. Biochem. Physiol. 122B, 223–233.

Nelson, D.R., Fatland, C.L., Buckner, J.S., Freeman, T.P., 1999b. External lipids of adults of the giant whitefly, *Aleurodicus dugesii*. Comp. Biochem. Physiol. 123B, 137–145.

Ogden, J., Pardo, C., Tchapla, A., 1998. Method development for the analysis of bee cuticular hydrocarbons and esters by capillary gas chromatography. J. Chromatogr. Sci. 36, 287–292.

Pomonis, J.G., Hakk, H., Fatland, C.L., 1989. Synthetic methyl- and dimethylalkanes: Kovats Indices, [^{13}C]NMR and mass spectra of some methylpentacosanes and 2,X-dimethylheptacosanes. J. Chem. Ecol. 15, 2319–2333.

Petroski, R.J., Bartelt, R.J., Vetter, R.S., 1994. Male-produced aggregation pheromone of *Carpophilus obsoletus* (Coleoptera: Nitidulidae). J. Chem. Ecol. 20, 1483–1493.

Provost, E., Riviere, G., Roux, M., Morgan, E.D., Bagneres, A.G., 1993. Change in chemical signature of the ant *Leptothorax lichtensteini* Bondroit with time. Insect Biochem. Mol. Biol. 23, 945–957.

Provost, E., Riviere, G., Roux, M., Bagneres, A.-G., Clement, J.L., 1994. Cuticular hydrocarbons whereby *Messor barbarus* ant workers putatively discriminate between monogynous and polygynous colonies. Are workers labeled by queens? J. Chem. Ecol. 20, 2985–3003.

Regnier, F.E., Nieh, M., Hölldobler, B., 1973. The volatile Dufour's gland components of the harvester ants *Pogonomyrmex rugosus* and *P. barbatus*. J. Insect Physiol. 19, 981–992.

de Renobales, M., Nelson, D.R., Mackay, M.E., Zamboni, A.C., Blomquist, G.J., 1988. Dynamics of hydrocarbon biosynthesis and transport to the cuticle during pupal and early adult development in the cabbage looper *Trichoplusia ni* (Lepidoptera: Noctuidae). Insect Biochem. 18, 607–613.

de Renobales, M., Nelson, D.R., Zamboni, A.C., Mackay, M.E., Dwyer, L.A., Theisen, M.O., Blomquist, G.J., 1989. Biosynthesis of very long-chain methyl-branched alcohols during pupal development in the cabbage looper, *Trichoplusia ni*. Insect Biochem. 19, 209–214.

Sutton, B.D., Carlson, D.A., 1997. Cuticular hydrocarbons of *Glossina*, III: Subgenera *Glossina* and *Nemorhina*. J. Chem. Ecol. 23, 1291–1320.

Takematsu, Y., Yamaoka, R., 1999. Cuticular hydrocarbons of *Reticulitermes* (Isoptera: Rhinotermitidae) in Japan and neighboring countries as chemotaxonomic characters. Appl. Entomol. Zool. 34, 179–188.

Thompson, M.J., Glancey, B.M., Robbins, W.E. et al., 1981. Major hydrocarbons of the post-pharyngeal glands of mated queens of the red imported fire ant *Solenopsis invicta*. Lipids 16, 485–495.